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(54) Title: IDENTIFICATION OF A cDNA ASSOCIATED WITH VENTRICULAR FUNCTION IN HUMAN MYOCARDIAL TISSUE

(57) Abstract: The invention relates generally to the changes in gene expression in myocardial tissue due to LVAD therapy for the treatment of cardiomyopathy and related symptoms. The invention relates specifically to a novel human gene which corresponds to a mRNA which is differentially regulated in myocardial biopsies taken from patients with LVAD implants. The invention includes methods for diagnosing and assessing cardiomyopathy and LVAD therapy by measuring the amount of gene product expressed in a patient. The invention also relates to methods of identifying agents which alter expression of the gene product of the invention and subsequent use of those agents to treat cardiomyopathy and facilitate LVAD therapy.

IDENTIFICATION OF A cDNA ASSOCIATED WITH VENTRICULAR FUNCTION IN HUMAN MYOCARDIAL TISSUE

FIELD OF THE INVENTION

The invention relates generally to the changes in gene expression in myocardial
5 tissue in patients with end-stage cardiomyopathy before and after surgical implantation of
a left ventricle assist device (LVAD). The invention relates specifically to a novel human
gene which is down-regulated in myocardial tissue following LVAD implantation in
human patients. This application claims priority to U.S. Provisional Application
60/184,825, filed February 24, 2000, which is herein incorporated by reference in its
10 entirety.

BACKGROUND OF THE INVENTION

Dilated cardiomyopathy is characterized by increased left ventricular or
biventricular dimensions with decreased ventricular ejection. Myocardial contractility is
severely impaired in systole along with variable impairment of relaxation and compliance
15 during diastole (Rockney, "Valvular heart disease" in: Bennett & Plum, Textbook of
Medicine - 20th edition, Saunders (1996) 319-327). Until recently, dilated
cardiomyopathy was considered an incurable, uniformly fatal chronic disease.
Epidemiologic studies, however, have demonstrated that at least a quarter of patients with
recent onset of symptoms with cardiomyopathy manifest spontaneous improvement and a
20 sustained favorable prognosis (Schocken *et al.*, J. Am. Coll. Cardiol. (1992) 20, 301-
306). Patients who present with new-onset cardiomyopathy must be managed medically
and closely followed. Transient mechanical support as a bridge to recovery is sometimes
recommended in certain patients before committing to cardiac transplantation.

Dilated cardiomyopathy and the decision to consider alternative surgical
25 procedures versus cardiac transplantation can be better assessed by studies at the
biochemical and molecular level. At present, new surgical procedures such as LVAD
(left ventricular assist devices) are promising therapies and potential alternatives to

cardiac transplantation. Approximately one-thousand people with end-stage cardiomyopathy have received LVAD as a bridge to cardiac transplantation since 1990 (McCarthy *et al.*, J. Thorac. Cardiovasc. Surg. (1991) 102, 578-587). These patients remain on the LVAD on average about one-hundred days before receiving a donor heart.

5 The LVAD implant operation is highly risky, in part, because the patients are so desperately ill. But those who survive the initial LVAD implantation surgery go on to do as well as other heart transplant patients. Overall, heart recipients have a four-year survival rate of greater than seventy percent (Kreitt *et al.*, J. Heart Lung Transplant. (1991) 10, 491-498).

10 The LVAD is a mechanical pump that is implanted below the diaphragm while the battery pack which powers the pump is carried outside the body. This device is surgically implanted in the patients' thoracic cavity and bypasses the failing left ventricle of the heart, maintaining the function of the heart to pump that is unable to effectively perform on its own. Patients awaiting a heart transplant often have to wait for extended
15 periods before a suitable heart becomes available. During this wait, the patient's already weakened heart may deteriorate and become unable to pump enough blood to sustain life. The LVAD assists the weakened heart allowing time for a donor heart to be obtained for the patient. As it is placed in patients whose hearts continue to fail, this device is an end-stage option for the patient presenting dilated cardiomyopathy and heart failure.

20 The typical LVAD has a tube going into the left ventricle that pulls blood from the ventricle into the pump. This pump then sends blood into the aorta and effectively bypasses the weakened ventricle. Another tube attached to the pump, is brought out of the wall of the abdomen, outside the body and attached to the control system and batteries to operate the device. LVADs are typically used for several weeks to months
25 but recent studies have examined the use of LVAD as a permanent therapy for end-stage cardiomyopathy (Oz *et al.*, Cardiac Chron. (1993) 7, 1-7).

The basis for dilated cardiomyopathy has been studied at the molecular level. Phenotypic heterogeneity, different patterns of transmission, and different frequencies of cardiac autoantibodies indicate that multiple genes and pathogenetic mechanisms can

lead to dilated cardiomyopathy (Mestroni *et al.*, J. Am. Coll. Cardiol. (1999) 34, 181-90). For instance, deletion of the entire dystrophin gene has been found to be associated with dilated cardiomyopathy in canines (Schatzberg *et al.*, Neuromuscul. Disord. (1999) 9, 289-95). In humans, terminally differentiated cardiomyocytes have the capacity to
5 synthesize new DNA and exhibit plasticity by a compensatory growth response following ischemic injury. The long-term effects of these compensatory responses may promote disease progression possibly due to the increased frequency of genetic mutations associated with increases in the synthesis of DNA. This concept has engaged scientists to investigate human models to explore the molecular and genetic basis of hypertrophy or
10 dilation of the myocardium.

The identification of new genes that are differentially expressed in heart tissue during dilated cardiomyopathy and following LVAD implantation will allow for the development of numerous diagnostic and therapeutic applications such as molecular probes and new agents which modulate the activity or expression of these genes.

15 SUMMARY OF THE INVENTION

The invention encompasses an isolated nucleic acid molecule that encodes the amino acid sequence of SEQ ID NO: 2; an isolated nucleic acid molecule that encodes a fragment of at least six amino acids of SEQ ID NO: 2; an isolated nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO: 1 under conditions
20 of sufficient stringency to produce a clear signal; and an isolated nucleic acid molecule which hybridizes to a nucleic acid molecule that encodes the amino acid sequence of SEQ ID NO: 2 under conditions of sufficient stringency to produce a clear signal. Nucleic acid molecules of the invention may have 60% nucleotide sequence identity through the open-reading frame of SEQ ID NO:1, preferably about 70-75% sequence
25 identity, more preferably about 80-85% sequence identity, and even more preferably at least about 90% sequence identity through the open reading frame.

The isolated nucleic acid molecule may be operably linked to one or more expression control elements, integrated into a vector and used to transform prokaryotic

hosts and eukaryotic host cells. The invention also includes a method for producing a polypeptide comprising the step of culturing a host cell transformed with the nucleic acid under conditions in which the protein encoded by said nucleic acid molecule is expressed.

5 The invention further includes an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 2; an isolated polypeptide comprising a fragment of at least six amino acids of SEQ ID NO: 2; an isolated polypeptide comprising conservative amino acid substitutions of SEQ ID NO: 2; and naturally occurring amino acid sequence variants of SEQ ID NO: 2. Also encompassed in the invention is an antibody that binds
10 to a polypeptide of the invention, such antibodies may be monoclonal or polyclonal.

 The invention encompasses a method of identifying an agent which modulates the expression of the polypeptide of the invention comprising the steps of: exposing cells which express the polypeptide to the agent; and determining whether the agent modulates expression of said polypeptide, thereby identifying an agent which modulates the
15 expression of the polypeptide.

 The invention further encompasses a method of identifying an agent which modulates the activity of the polypeptide of the invention comprising the steps of: exposing cells which express the polypeptide to the agent; and determining whether the agent modulates the activity of said polypeptide, thereby identifying an agent which
20 modulates the activity of the polypeptide.

 The invention also encompasses a method of identifying an agent which modulates the transcription of the nucleic acid molecule of the invention comprising the steps of: exposing cells which transcribe the nucleic acid to the agent; and determining whether the agent modulates transcription of said nucleic acid, thereby identifying an
25 agent which modulates the transcription of the nucleic acid.

 Also embodied in the invention is a method of identifying binding partners for the polypeptide of the invention comprising the steps of: exposing the polypeptide to a potential binding partner; and determining if the potential binding partner binds to the polypeptide, thereby identifying binding partners for the polypeptide.

Methods for modulating the expression or activity of a nucleic acid or polypeptide of the invention comprising administering an effective amount of an agent which modulates the expression or activity of a nucleic acid or polypeptide are also encompassed in the invention.

5 In yet another embodiment, a non-human transgenic animal modified to contain a nucleic acid molecule of the invention is contemplated. The nucleic acid molecule may also contain a mutation(s) that prevents production of functional protein, constitutively activates the protein or constitutes a dominant negative mutant.

The invention encompasses a method of identifying an agent which modulates the
10 amount of gene product expressed from the nucleic acid of the invention comprising the steps of: administering the agent to be tested to a mammal; and measuring the amount of the gene product expressed in the tissue of said mammal; wherein an increase or decrease in the level of the gene product expressed identifies an agent capable of modulating expression of said gene product.

15 The invention also includes methods for diagnosing cardiomyopathy comprising the step of determining the level of expression of the nucleic acid or polypeptide of the invention.

Also included are methods for preventing or treating cardiomyopathy comprising administering a therapeutically effective amount of the nucleic acid or polypeptide of the
20 invention. Such methods may diminish the occurrence of at least one of the following symptoms associated with cardiomyopathy; reduced ejection fraction, increased left ventricular diastolic dimension, decreased ventricular wall thickness, increased atrial size, valvular regurgitation, exertional intolerance and ventricular tachyarrhythmia.

The invention further includes a method of assessing the effect of LVAD for
25 treatment of cardiomyopathy comprising measuring the amount of the polypeptide of the invention expressed wherein an alteration in the amount of the polypeptide expressed correlates to successful LVAD treatment. Another method encompassed in the invention includes altering the amount of time a patient can remain on LVAD treatment by modulating the amount of the polypeptide of the invention expressed in myocardial

tissue.

The invention also encompasses a method of identifying patients who require LVAD treatment by measuring the amount of gene product from the nucleic acid of the invention expressed in myocardial tissue wherein an alteration in the amount of said gene product expressed is indicative of a patient requiring LVAD treatment.

The invention further encompasses a method of preventing or treating cardiomyopathy comprising modulating the amount of gene product from the nucleic acid of the invention expressed in myocardial tissue by administering a therapeutically effective amount of the agent identified using the methods of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 READS™ (Restriction Enzyme Analysis of Differentially expressed Sequences) results displaying regulation of cardiomyopathy associated protein (14431) (SEQ ID NO: 1) expression in myocardial biopsies from normal hearts and myocardial biopsies prior to LVAD therapy and following cardiectomy prior to cardiac transplantation.

Figure 2 Quantitative RT-PCR results displaying 14431 (SEQ ID NO: 1) mRNA expression in myocardial biopsies from normal hearts and myocardial biopsies prior to LVAD therapy and following cardiectomy prior to cardiac transplantation.

Figure 3 Expanded quantitative RT-PCR results displaying 14431 (SEQ ID NO: 1) mRNA expression in myocardial biopsies from normal hearts and myocardial biopsies prior to LVAD therapy and following cardiectomy prior to cardiac transplantation.

Figure 4 Extended quantitative RT-PCR results displaying 14431 (SEQ ID NO: 1) mRNA expression in myocardial tissue from normal hearts, hearts from patients suffering from ischemic cardiomyopathy (IsCM), idiopathic cardiomyopathy (IDCM), valvular disease (valvular) or other heart disease (other).

Figure 5 Quantitative RT-PCR tissue profile panel from kidney, adrenal gland, pancreas, salivary gland, liver, prostate, thyroid, cerebellum, fetal brain, placenta, spinal cord, stomach, small intestine, bone marrow, thymus, spleen, heart, lung, testes, uterus,

mammary gland and trachea.

Figure 6 Predicted hydrophobicity, hydrophilicity, antigenic index and structure plots for SEQ ID NO: 2.

Figure 7 Northern blot of various tissues.

5

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

I. General Description

The present invention is based in part on a new gene that is differentially expressed in human LVAD myocardial biopsy samples. The human gene encodes a protein of 358 amino acids designated as cardiomyopathy associated protein (CAP).

The protein can serve as a target for agents that can be used to modulate the expression or activity of the proteins. For example, agents may be identified which modulate biological processes associated with cardiomyopathy, LVAD therapy and cardiac transplantation.

15

II. Specific Embodiments

A. The Protein Associated with Cardiomyopathy

The present invention provides isolated protein, allelic variants of the protein, and conservative amino acid substitutions of the protein. As used herein, "protein" or polypeptide refers to a protein that has the human amino acid sequence depicted in SEQ ID NO: 2 as well as the related molecules described herein. The invention also includes naturally occurring allelic variants and proteins that have a slightly different amino acid sequence than that specifically recited above. Allelic variants, though possessing a slightly different amino acid sequence than those recited above, will still have the same or similar biological functions associated with the 358 amino acid protein.

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As used herein, the family of proteins related to the human amino acid sequence of SEQ ID NO: 2 refers to proteins that have been isolated from organisms in addition to humans. The methods used to identify and isolate other members of the family of proteins related to the 358 amino acid protein are described below.

The proteins of the present invention are preferably in isolated form. As used herein, a protein is said to be isolated when physical, mechanical or chemical methods are employed to remove the protein from cellular constituents that are normally associated with the protein. A skilled artisan can readily employ standard purification methods to
5 obtain an isolated protein.

The proteins of the present invention further include conservative amino acid substitution variants (*i.e.*, conservative) of the proteins herein described. As used herein, a conservative variant refers to at least one alteration in the amino acid sequence that does not adversely affect the biological functions of the protein. A substitution, insertion
10 or deletion is said to adversely affect the protein when the altered sequence prevents or disrupts a biological function associated with the protein. For example, the overall charge, structure or hydrophobic/hydrophilic properties of the protein can be altered without adversely affecting a biological activity. Accordingly, the amino acid sequence can often be altered, for example to render the peptide more hydrophobic or hydrophilic,
15 without adversely affecting the biological activities of the protein.

Ordinarily, the allelic variants, the conservative substitution variants, and the members of the protein family, will have an amino acid sequence having at least about 50%, 60%, 70% or 75% amino acid sequence identity with the sequence set forth in SEQ ID NO: 2 more preferably at least about 80%, even more preferably at least about 90%,
20 and most preferably at least about 95%. Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. N-terminal,
25 C-terminal or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

Thus, the proteins of the present invention include molecules having the amino acid sequence disclosed in SEQ ID NO: 2; fragments thereof having a consecutive sequence of at least about 3, 4, 5, 6, 10, 15, 20, 25, 30, 35 or more amino acid residues of

the proteins, for instance, antigenic fragments (see Figure 6); amino acid sequence variants wherein an amino acid residue has been inserted N- or C-terminal to, or within, the disclosed sequence; and amino acid sequence variants of the disclosed sequence, or their fragments as defined above, that have been substituted by another residue.

- 5 Contemplated variants further include those containing predetermined mutations by, *e.g.*, homologous recombination, site-directed or PCR mutagenesis, and the corresponding proteins of other animal species, including but not limited to rabbit, mouse, rat, porcine, bovine, ovine, equine and non-human primate species, and the alleles or other naturally occurring variants of the family of proteins; and derivatives wherein the protein has been
- 10 covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope).

- As described below, members of the family of proteins can be used: 1) to identify agents which modulate at least one activity of the protein; 2) to identify binding partners
- 15 for the protein, 3) as an antigen to raise polyclonal or monoclonal antibodies, 4) as a therapeutic agent or target and 5) as a diagnostic marker.

B. Nucleic Acid Molecules

- The present invention further provides a nucleic acid molecule which encodes the
- 20 protein having SEQ ID NO: 2, and the related proteins herein described, preferably in isolated form. As used herein, "nucleic acid" includes RNA or DNA molecules that encode a protein or peptide as defined above, is complementary to a nucleic acid sequence encoding such peptides, hybridizes to such a nucleic acid and remains stably bound to it under appropriate stringency conditions, encodes a polypeptide sharing at
- 25 least about 75% sequence identity, preferably at least 80%, and more preferably at least about 85%, with the peptide sequences or exhibits at least about 60% nucleotide sequence identity over nucleotides 283-1356 of SEQ ID NO:1, preferably about 70-75% sequence identity, more preferably about 80-85% sequence identity or more preferably, about 90-95 sequence identity through the open reading frame defined by nucleotide 285-

1356. Specifically contemplated are genomic DNA, cDNA, mRNA and antisense molecules, as well as nucleic acids based on alternative backbones or including alternative bases whether derived from natural sources or synthesized. Such hybridizing or complementary nucleic acids, however, are defined further as being novel and non-obvious over any prior art nucleic acid including that which encodes, hybridizes under appropriate stringency conditions, or is complementary to nucleic acid encoding a protein according to the present invention.

Homology or identity at the amino acid or nucleotide level is determined by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn and tblastx (Karlin *et al.*, Proc. Natl. Acad. Sci. USA (1990) 87, 2264-2268 and Altschul, J. Mol. Evol. (1993) 36, 290-300, fully incorporated by reference) which are tailored for sequence similarity searching. The approach used by the BLAST program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.*, (Nature Genetics (1994) 6, 119-129) which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (*i.e.*, the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff *et al.*, Proc. Natl. Acad. Sci. USA, (1992) 89, 10915-10919, fully incorporated by reference). For blastn, the scoring matrix is set by the ratios of M (*i.e.*, the reward score for a pair of matching residues) to N (*i.e.*, the penalty score for mismatching residues), wherein the default values for M and N are 5 and -4, respectively.

"Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1%

polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5× Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2× SSC and 0.1% SDS. A skilled artisan can readily determine and vary the stringency conditions appropriately to obtain a clear and detectable hybridization signal. Preferred molecules are those that hybridize under the above conditions to the complements of SEQ ID NO: 1 and which encode a functional protein.

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid.

The present invention further provides fragments of the encoding nucleic acid molecule. As used herein, a fragment of an encoding nucleic acid molecule refers to a small portion of the entire protein coding sequence. The size of the fragment will be determined by the intended use. For example, if the fragment is chosen so as to encode an active portion of the protein, the fragment will need to be large enough to encode the functional region(s) of the protein. For instance, fragments of the invention encode antigenic fragments of the protein having SEQ ID NO: 2 as set forth in Figure 6. If the fragment is to be used as a nucleic acid probe or PCR primer, then the fragment length is chosen so as to obtain a relatively small number of false positives during probing and priming.

Fragments of the encoding nucleic acid molecules of the present invention (*i.e.*, synthetic oligonucleotides) that are used as probes or specific primers for the polymerase chain reaction (PCR), or to synthesize gene sequences encoding proteins of the invention can easily be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci *et al.*, J. Am. Chem. Soc. (1981) 103, 3185-3191) or using automated synthesis methods. In addition, larger DNA segments can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the gene, followed by ligation of oligonucleotides to build the complete modified gene.

The encoding nucleic acid molecules of the present invention may further be modified so as to contain a detectable label for diagnostic and probe purposes. A variety of such labels are known in the art and can readily be employed with the encoding molecules herein described. Suitable labels include, but are not limited to, biotin, radiolabeled nucleotides and the like. A skilled artisan can employ any of the art known labels to obtain a labeled encoding nucleic acid molecule.

Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence encoded by a nucleic acid falling within the contemplated scope of the present invention.

C. Isolation of Other Related Nucleic Acid Molecules

As described above, the identification and characterization of the human nucleic acid molecule having SEQ ID NO: 1 allows a skilled artisan to isolate other members of the protein family in addition to the sequences herein described. Further, the presently disclosed nucleic acid molecules allow a skilled artisan to isolate nucleic acid molecules that encode other members of the family of proteins in addition to the protein having SEQ ID NO: 2.

Essentially, a skilled artisan can readily use the amino acid sequence of SEQ ID NO: 2, to generate antibody probes to screen expression libraries prepared from appropriate cells. Typically, polyclonal antiserum from mammals such as rabbits immunized with the purified protein (as described below) or monoclonal antibodies can be used to probe a mammalian cDNA or genomic expression library, such as lambda gtl library, to obtain the appropriate coding sequence for other members of the protein family. The cloned cDNA sequence can be expressed as a fusion protein, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme.

Alternatively, a portion of the coding sequence herein described can be synthesized and used as a probe to retrieve DNA encoding a member of the protein family from any

mammalian organism. Oligomers containing approximately 18-20 nucleotides (encoding about a 6-7 amino acid stretch) are prepared and used to screen genomic DNA or cDNA libraries to obtain hybridization under stringent conditions or conditions of sufficient stringency to eliminate an undue level of false positives.

- 5 Additionally, pairs of oligonucleotide primers can be prepared for use in a polymerase chain reaction (PCR) to selectively clone an encoding nucleic acid molecule. A PCR denature/anneal/extend cycle for using such PCR primers is well known in the art and can readily be adapted for use in isolating other encoding nucleic acid molecules.

10 D. rDNA molecules containing a DNA molecule

The present invention further provides recombinant DNA molecules (rDNAs) that contain a coding sequence. As used herein, a rDNA molecule is a DNA molecule that has been subjected to molecular manipulation *in situ*. Methods for generating rDNA molecules are well known in the art, for example, see Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1985). In the preferred rDNA molecules, a coding DNA sequence is operably linked to expression control sequences and/or vector sequences.

- 15 The choice of vector and expression control sequences to which one of the protein family encoding sequences of the present invention is operably linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, protein expression, and the host cell to be transformed. A vector contemplated by the present invention is at least capable of directing the replication or insertion into the host chromosome, and preferably also expression, of the structural gene included in the rDNA molecule.

- 20 Expression control elements that are used for regulating the expression of an operably linked protein encoding sequence are known in the art and include, but are not limited to, inducible promoters, constitutive promoters, secretion signals, and other regulatory elements. Preferably, the inducible promoter is readily controlled, such as being responsive to a nutrient in the host cell's medium.

In one embodiment, the vector containing a coding nucleic acid molecule will include

a prokaryotic replicon, *i.e.*, a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extra-chromosomally in a prokaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, vectors that include a prokaryotic replicon may also include a gene whose expression confers a detectable marker such as drug resistance. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Vectors that include a prokaryotic replicon can further include a prokaryotic or bacteriophage promoter capable of directing the expression (transcription and translation) of the coding gene sequences in a bacterial host cell, such as *E. coli*. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from BioRad Laboratories (Richmond, CA), pPL and pKK223 available from Pharmacia (Piscataway, NJ).

Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells such as myocardial cells, can also be used to form a rDNA molecules that contains a coding sequence. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1/pML2d (International Biotechnologies, Inc.), pTDT1 (ATCC, #31255), the vector pCDM8 described herein, and the like eukaryotic expression vectors. Vectors may be modified to include myocardial cell specific promoters if needed.

Eukaryotic cell expression vectors used to construct the rDNA molecules of the present invention may further include a selectable marker that is effective in an eukaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, *i.e.*, the neomycin phosphotransferase

(*neo*) gene (Southern *et al.*, J. Mol. Appl. Genet. (1982) 1, 327-341). Alternatively, the selectable marker can be present on a separate plasmid, and the two vectors are introduced by co-transfection of the host cell, and selected by culturing in the appropriate drug for the selectable marker.

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B. Host Cells Containing an Exogenously Supplied Coding Nucleic Acid

The present invention further provides host cells transformed with a nucleic acid molecule that encodes a protein of the present invention. The host cell can be either prokaryotic or eukaryotic. Eukaryotic cells useful for expression of a protein of the invention are not limited, so long as the cell line is compatible with cell culture methods and compatible with the propagation of the expression vector and expression of the gene product. Preferred eukaryotic host cells include, but are not limited to, yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human cell line. Preferred eukaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells (NIH3T3) available from the ATCC as CRL 1658, baby hamster kidney cells (BHK), and the like eukaryotic tissue culture cell lines.

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Any prokaryotic host can be used to express a rDNA molecule encoding a protein of the invention. The preferred prokaryotic host is *E. coli*.

Transformation of appropriate cell hosts with a rDNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used and host system employed. With regard to transformation of prokaryotic host cells, electroporation and salt treatment methods are typically employed, see, for example, Cohen *et al.*, Proc. Natl. Acad. Sci. USA (1972) 69, 2110-2114; and Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1982). With regard to transformation of vertebrate cells with vectors containing rDNAs, electroporation, cationic lipid or salt treatment methods are typically employed, see, for example, Graham *et al.*, Virology (1973) 52, 456-467; and Wigler *et al.*, Proc. Natl. Acad. Sci. USA (1979) 76, 1373-1376.

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Successfully transformed cells, *i.e.*, cells that contain a rDNA molecule of the present

invention, can be identified by well known techniques including the selection for a selectable marker. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to produce single colonies. Cells from those colonies can be harvested, lysed and their DNA content examined for the presence of the rDNA using a
5 method such as that described by Southern, J. Mol. Biol. (1975) 98, 503-517; or Berent *et al.*, Biotech. Histochem. (1985) 3, 208; or the proteins produced from the cell assayed via an immunological method.

F. Production of Recombinant Proteins using a rDNA Molecule

10 The present invention further provides methods for producing a protein of the invention using nucleic acid molecules herein described. In general terms, the production of a recombinant form of a protein typically involves the following steps: First, a nucleic acid molecule is obtained that encodes a protein of the invention, such as the nucleic acid molecule depicted in SEQ ID NO: 1 or nucleotides 283-1356 of SEQ ID NO: 1. If the encoding
15 sequence is uninterrupted by introns as is SEQ ID NO: 1, it is directly suitable for expression in any host. The nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the protein open reading frame. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the recombinant
20 protein. Optionally the recombinant protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in
25 appropriate hosts. The construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so

as to provide an excisable gene to insert into these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with the nucleic acid molecules of the invention to produce recombinant protein.

5 G. Methods to Identify Binding Partners

Another embodiment of the present invention provides methods for isolating and identifying binding partners of proteins of the invention. In detail, a protein of the invention is mixed with a potential binding partner or an extract or fraction of a cell under conditions that allow the association of potential binding partners with the protein of the invention. After
10 mixing, peptides, polypeptides, proteins or other molecules that have become associated with a protein of the invention are separated from the mixture. The binding partner that bound to the protein of the invention can then be removed and further analyzed. To identify and isolate a binding partner, the entire protein, for instance a protein comprising the entire amino acid sequence of SEQ ID NO: 2 can be used. Alternatively, a fragment of the protein can be used.

15 As used herein, a cellular extract refers to a preparation or fraction which is made from a lysed or disrupted cell. The preferred source of cellular extracts will be cells derived from human cardiac tissue, for instance, LVAD biopsy tissue or tissue culture cells.

A variety of methods can be used to obtain an extract of a cell. Cells can be disrupted using either physical or chemical disruption methods. Examples of physical disruption
20 methods include, but are not limited to, sonication and mechanical shearing. Examples of chemical lysis methods include, but are not limited to, detergent lysis and enzyme lysis. A skilled artisan can readily adapt methods for preparing cellular extracts in order to obtain extracts for use in the present methods.

Once an extract of a cell is prepared, the extract is mixed with the protein of the
25 invention under conditions in which association of the protein with the binding partner can occur. A variety of conditions can be used, the most preferred being conditions that closely resemble conditions found in the cytoplasm of a human cell. Features such as osmolarity, pH, temperature, and the concentration of cellular extract can be varied to optimize the association of the protein with the binding partner.

After mixing under appropriate conditions, the bound complex is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For example, antibodies specific to a protein of the invention can be used to immunoprecipitate the binding partner complex. Alternatively, standard chemical separation techniques such as chromatography and density/sediment centrifugation can be used.

After removal of non-associated cellular constituents found in the extract, the binding partner can be dissociated from the complex using conventional methods. For example, dissociation can be accomplished by altering the salt concentration or pH of the mixture.

To aid in separating associated binding partner pairs from the mixed extract, the protein of the invention can be immobilized on a solid support. For example, the protein can be attached to a nitrocellulose matrix or acrylic beads. Attachment of the protein to a solid support aids in separating peptide/binding partner pairs from other constituents found in the extract. The identified binding partners can be either a single protein or a complex made up of two or more proteins. Alternatively, binding partners may be identified using a Far-Western assay according to the procedures of Takayama *et al.*, Methods Mol. Biol. (1997) 69, 171-184 or identified through the use of epitope tagged proteins or GST fusion proteins.

Alternatively, the nucleic acid molecules of the invention can be used in a yeast two-hybrid system. The yeast two-hybrid system has been used to identify other protein partner pairs and can readily be adapted to employ the nucleic acid molecules herein described.

H. Methods to Identify Agents that Modulate the Expression a Nucleic Acid Encoding the Cardiomyopathy Associated Protein

Another embodiment of the present invention provides methods for identifying agents that modulate the expression of a nucleic acid encoding a protein of the invention such as a protein having the amino acid sequence of SEQ ID NO: 2. Such assays may utilize any available means of monitoring for changes in the expression level of the nucleic acids of the invention. As used herein, an agent is said to modulate the expression of a nucleic acid of the invention, for instance a nucleic acid encoding the protein having the sequence of SEQ ID NO: 2, if it is capable of up- or down-regulating expression of the nucleic acid in a cell.

In one assay format, cell lines that contain reporter gene fusions between any region of the open reading frame defined by nucleotides 283-1356 of SEQ ID NO: 1 or fragments under control of the gene's promoter and any assayable fusion partner may be prepared. Numerous assayable fusion partners are known and readily available including the firefly luciferase gene and the gene encoding chloramphenicol acetyltransferase (Alam *et al.*, Anal. Biochem. (1990) 188, 245-254). Cell lines containing the reporter gene fusions are then exposed to the agent to be tested under appropriate conditions and time. Differential expression of the reporter gene between samples exposed to the agent and control samples identifies agents which modulate the expression of a nucleic acid encoding the protein having the sequence of SEQ ID NO: 2.

Additional assay formats may be used to monitor the ability of the agent to modulate the expression of a nucleic acid encoding a protein of the invention such as the protein having SEQ ID NO: 2. For instance, mRNA expression may be monitored directly by hybridization to the nucleic acids of the invention. Cell lines are exposed to the agent to be tested under appropriate conditions and time and total RNA or mRNA is isolated by standard procedures such those disclosed in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1985).

Probes to detect differences in RNA expression levels between cells exposed to the agent and control cells may be prepared from the nucleic acids of the invention. It is preferable, but not necessary, to design probes which hybridize only with target nucleic acids under conditions of high stringency. Only highly complementary nucleic acid hybrids form under conditions of high stringency. Accordingly, the stringency of the assay conditions determines the amount of complementarity which should exist between two nucleic acid strands in order to form a hybrid. Stringency should be chosen to maximize the difference in stability between the probe:target hybrid and potential probe:non-target hybrids.

Probes may be designed from the nucleic acids of the invention through methods known in the art. For instance, the G+C content of the probe and the probe length can affect probe binding to its target sequence. Methods to optimize probe specificity are commonly available in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor

Laboratory Press (1985); or Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publishing Company (1995).

Hybridization conditions are modified using known methods, such as those described by Sambrook *et al.* and Ausubel *et al.* as required for each probe. Hybridization of total
5 cellular RNA or RNA enriched for polyA RNA can be accomplished in any available format. For instance, total cellular RNA or RNA enriched for polyA RNA can be affixed to a solid support and the solid support exposed to at least one probe comprising at least one, or part of one of the sequences of the invention under conditions in which the probe will specifically hybridize. Alternatively, nucleic acid fragments comprising at least one, or part of one of the
10 sequences of the invention can be affixed to a solid support, such as a porous glass wafer. The glass wafer can then be exposed to total cellular RNA or polyA RNA from a sample under conditions in which the affixed sequences will specifically hybridize. Such glass wafers and hybridization methods are widely available, for example, those disclosed by Beattie (WO 95/11755). By examining for the ability of a given probe to specifically
15 hybridize to an RNA sample from an untreated cell population and from a cell population exposed to the agent, agents which up or down regulate the expression of a nucleic acid encoding the protein having the sequence of SEQ ID NO: 2 are identified.

Hybridization for qualitative and quantitative analysis of mRNA may also be carried out by using a RNase Protection Assay (*i.e.*, RPA, see Ma *et al.*, Methods (1996) 10, 273-
20 238). Briefly, an expression vehicle comprising cDNA encoding the gene product and a phage specific DNA dependent RNA polymerase promoter (*e.g.*, T7, T3 or SP6 RNA polymerase) is linearized at the 3' end of the cDNA molecule, downstream from the phage promoter, wherein such a linearized molecule is subsequently used as a template for synthesis of a labeled antisense transcript of the cDNA by *in vitro* transcription. The labeled transcript
25 is then hybridized to a mixture of isolated RNA (*i.e.*, total or fractionated mRNA) by incubation at 45°C overnight in a buffer comprising 80% formamide, 40 mM Pipes, pH 6.4, 0.4 M NaCl and 1 mM EDTA. The resulting hybrids are then digested in a buffer comprising 40 µg/ml ribonuclease A and 2 µg/ml ribonuclease. After deactivation and extraction of extraneous proteins, the samples are loaded onto urea/polyacrylamide gels for analysis.

In another assay format, agents which effect the expression of the instant gene products, cells or cell lines would first be identified which express said gene products physiologically. Cells and cell lines so identified, such as cells derived from the spleen or testes (see Fig. 5), would be expected to comprise the necessary cellular machinery such that the fidelity of modulation of the transcriptional apparatus is maintained with regard to exogenous contact of agent with appropriate surface transduction mechanisms and/or the cytosolic cascades. Further, such cells or cell lines would be transduced or transfected with an expression vehicle (*e.g.*, a plasmid or viral vector) construct comprising an operable non-translated 5'-promoter upstream of the structural gene encoding the instant gene products fused to one or more antigenic fragments, which are peculiar to the instant gene products, wherein said fragments are under the transcriptional control of said promoter and are expressed as polypeptides whose molecular weight can be distinguished from the naturally occurring polypeptides or may further comprise an immunologically distinct tag. Such a process is well known in the art (see Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1982)).

Cells or cell lines transduced or transfected as outlined above would then be contacted with agents under appropriate conditions; for example, the agent comprises a pharmaceutically acceptable excipient and is contacted with cells comprised in an aqueous physiological buffer such as phosphate buffered saline (PBS) at physiological pH, Eagles balanced salt solution (BSS) at physiological pH, PBS or BSS comprising serum or conditioned media comprising PBS or BSS and/or serum incubated at 37°C. Said conditions may be modulated as deemed necessary by one of skill in the art. Subsequent to contacting the cells with the agent, said cells will be disrupted and the polypeptides from disrupted cells are fractionated such that a polypeptide fraction is pooled and contacted with an antibody to be further processed by immunological assay (*e.g.*, ELISA, immunoprecipitation or Western blot). The pool of proteins isolated from the "agent contacted" sample will be compared with a control sample where only the excipient is contacted with the cells and an increase or decrease in the immunologically generated signal from the "agent contacted" sample compared to the control will be used to distinguish the effectiveness of the agent.

I. Methods to Identify Agents that Modulate at Least One Activity of CAP

Another embodiment of the present invention provides methods for identifying agents that modulate at least one activity of a protein of the invention such as the protein having the amino acid sequence of SEQ ID NO: 2. Such methods or assays may utilize any means of
5 monitoring or detecting the desired activity.

In one format, the relative amounts of a protein of the invention between a cell population that has been exposed to the agent to be tested compared to an un-exposed control cell population may be assayed. In this format, probes such as specific antibodies are used to monitor the differential expression of the protein in the different cell populations. Cell lines
10 or populations are exposed to the agent to be tested under appropriate conditions and time. Cellular lysates may be prepared from the exposed cell line or population and a control, unexposed cell line or population. The cellular lysates are then analyzed with the probe.

Antibody probes are prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptides, polypeptides or proteins of the
15 invention if they are of sufficient length, or, if desired, or if required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical
20 Co. (Rockford, IL), may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy terminus with a cysteine residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the
25 immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared

using the standard method of Kohler and Milstein (Nature (1975) 256, 495-497) or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten, polypeptide or protein. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either *in vitro* or by production in ascites fluid.

The desired monoclonal antibodies are then recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonal or polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab', of F(ab')₂ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Antibody regions that bind specifically to the desired regions of the protein can also be produced in the context of chimeras with multiple species origin, particularly humanized antibodies.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the a protein of the invention alone or with its associated substrates, binding partners, etc. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a non-random basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. For example, agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up predicted functional sites. Such sites include a zinc finger motif starting at residue 37 (VVILP CQHNLCKRCKA NDVFQ).

The agents of the present invention can be, as examples, peptides, small molecules,

vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention. Dominant negative proteins, DNA encoding these proteins, antibodies to these proteins, peptide fragments of these proteins or mimics of these proteins may be introduced into cells to affect function. "Mimic" used herein refers to the modification of a region or several regions of a peptide molecule to provide a structure chemically different from the parent peptide but topographically and functionally similar to the parent peptide (see Grant GA. in: Meyers (ed.) Molecular Biology and Biotechnology, VCH Publishers, (1995) 659-664).

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents of the present invention are antibodies immunoreactive with critical positions of proteins of the invention. Antibody agents are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the protein intended to be targeted by the antibodies.

J. Uses for Agents that Modulate at Least One Activity of the CAP

As provided in the Examples, the proteins and nucleic acids of the invention, such as the protein having the amino acid sequence of SEQ ID NO: 2 is expressed in myocardial tissue. Agents that modulate, up-or-down-regulate the expression of the protein or agents such as agonists or antagonists of at least one activity of the protein may be used to modulate biological and pathologic processes associated with the protein's function and activity.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by a protein of the invention. The term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

Pathological processes refer to a category of biological processes which produce a deleterious effect. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, cardiomyopathy may be prevented or disease progression modulated by the administration of agents which up-regulate or modulate in some way the expression or at least one activity of a protein of the invention.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. For example, an agent of the present invention can be administered in combination with other known drugs such as ACE inhibitors, digitalis glycosides, diuretics, vasodilators and β -blockers used in the treatment of cardiomyopathy or anti-rejection drugs used during transplantation. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents which modulate expression or at least one activity of a protein of the invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 $\mu\text{g/kg}$ body wt. The preferred dosages comprise 0.1 to 10 $\mu\text{g/kg}$ body weight. The most preferred dosages comprise 0.1 to 1 $\mu\text{g/kg}$ body weight.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble

form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be co-administered along with other compounds typically prescribed for these conditions according to generally accepted medical practice. The compounds of this invention can be utilized *in vivo*, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice, or *in vitro*.

K. Diagnosis of Cardiomyopathy by Measuring CAP Expression

One means of diagnosing cardiomyopathy or assessing the effects of LVAD implantation using the nucleic acid molecules or proteins of the invention involves obtaining myocardial tissue from living subjects. Obtaining tissue samples from living sources is problematic for tissues such as heart. However, due to the nature of the treatment paradigms for LVAD patients, biopsy material may be available. If possible, myocardial biopsy tissue may be obtained during LVAD implantation or removal.

The use of molecular biological tools has become routine in forensic technology. For example, nucleic acid probes may be used to determine the expression of a nucleic acid molecule comprising all or at least part of the sequence of SEQ ID NO: 1 in forensic or pathology specimens. Further, nucleic acid assays may be carried out by any means of conducting a transcriptional profiling analysis. In addition to nucleic acid analysis, forensic methods of the invention may target the protein encoded by SEQ ID NO: 1 to assay for the up or down regulation of its gene (Shiverick *et al.*, Biochim. Biophys. Acta (1975) 393, 124-133).

Methods of the invention may involve treatment of tissues with collagenases or other proteases to make the tissue amenable to cell lysis (Semenov *et al.*, Biull. Eksp. Biol. Med. (1987) 104, 113-116). Further, it is possible to obtain biopsy samples from different regions of the heart for analysis.

Assays to detect nucleic acid or protein molecules of the invention may be in any available format. Typical assays for nucleic acid molecules include hybridization or PCR based formats. Typical assays for the detection of proteins, polypeptides or peptides of the invention include the use of antibody probes in any available format such as *in situ* binding assays, etc. See Harlow *et al.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988). In preferred embodiments, assays are carried-out with appropriate controls.

L. Transgenic Animals

Transgenic animals containing mutant, knock-out, modified genes, or dominant negative mutants corresponding to the cDNA sequence of SEQ ID NO: 1 are also included in the invention. Transgenic animals are genetically modified animals into which recombinant, exogenous or cloned genetic material has been experimentally transferred. Such genetic material is often referred to as a "transgene". The nucleic acid sequence of the transgene, in this case a form of SEQ ID NO: 1, may be integrated either at a locus of a genome where that particular nucleic acid sequence is not otherwise normally found or at the normal locus for the transgene. The transgene may consist of nucleic acid sequences derived from the genome of

the same species or of a different species than the species of the target animal.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic alteration or genetic information was introduced into a germ line cell, thereby conferring the ability of the transgenic animal to transfer the genetic information to offspring.

- 5 If such offspring in fact possess some or all of that alteration or genetic information, then they too are transgenic animals.

The alteration or genetic information may be foreign to the species of animal to which the recipient belongs, foreign only to the particular individual recipient, or may be genetic information already possessed by the recipient. In the last case, the altered or introduced gene
10 may be expressed differently than the native gene.

Transgenic animals can be produced by a variety of different methods including transfection, electroporation, microinjection, gene targeting in embryonic stem cells and recombinant viral and retroviral infection (see, *e.g.*, U.S. Patents 4,736,866 & 5,602,307; Mullins *et al.*, Hypertension (1993) 22, 630-633; Brenin *et al.*, Surg. Oncol. (1997) 6, 99-110;
15 "Recombinant Gene Expression Protocols" in: Tuan (ed.), Methods in Molecular Biology, Humana Press, 1997)).

A number of recombinant or transgenic mice have been produced, including those which express an activated oncogene sequence (U.S. Patent 4,736,866); express simian SV40 T-antigen (U.S. Patent 5,728,915); lack the expression of interferon regulatory factor 1 (IRF-
20 1) (U.S. Patent 5,731,490); exhibit dopaminergic dysfunction (U.S. Patent 5,723,719); express at least one human gene which participates in blood pressure control (U.S. Patent 5,731,489); display greater similarity to the conditions existing in naturally occurring Alzheimer's disease (U.S. Patent 5,720,936); have a reduced capacity to mediate cellular adhesion (U.S. Patent 5,602,307); possess a bovine growth hormone gene (Clutter *et al.*,
25 Genetics (1996) 143, 1753-1760); or, are capable of generating a fully human antibody response (Bruggemann *et al.*, Curr. Opin. Biotechnol. (1997) 8, 455-458).

While mice and rats remain the animals of choice for most transgenic experimentation, in some instances it is preferable or even necessary to use alternative animal species. Transgenic procedures have been successfully utilized in a variety of non-murine

animals, including sheep, goats, pigs, dogs, cats, monkeys, chimpanzees, hamsters, rabbits, cows and guinea pigs (see, e.g., Kim *et al.*, Mol. Reprod. Dev. (1997) 46, 515-526; Houdebine, Reprod. Nutr. Dev. (1995) 35, 609-617; Petters, Reprod. Fertil. Dev. (1994) 6, 643-645; Schmieke *et al.*, Science (1997) 278, 2130-2133; and Amoah, J. Anim. Sci. (1997) 5 75, 578-585.

The method of introduction of nucleic acid fragments into recombination competent mammalian cells can be by any method which favors co-transformation of multiple nucleic acid molecules. Detailed procedures for producing transgenic animals are readily available to one skilled in the art, including the disclosures in U.S. Patent 5,489,743 and U.S. Patent 10 5,602,307.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present 15 invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1: Identification of Differentially Expressed CAP mRNA

Myocardial biopsies were obtained from patients prior to LVAD implantation and following cardiectomy prior to cardiac transplantation. Total cellular RNA was prepared from the biopsies described above as well as from control, non-ischemic donor heart tissue using the procedure of Newburger *et al.*, J. Biol. Chem. (1981) 266, 16171-16177 and Newburger *et al.*, Proc. Natl. Acad. Sci. USA (1988) 85, 5215-5219.

Synthesis of cDNA was performed as previously described by Prashar *et al.*, in WO 97/05286 (1997) and in Prashar *et al.*, Proc. Natl. Acad. Sci. USA (1996) 93, 659-663.

Briefly, cDNA was synthesized according to the protocol described in the Gibco-BRL kit for cDNA synthesis. The reaction mixture for first-strand synthesis included 6 µg of total RNA, and 200 ng of a mixture of one base anchored oligo(dT) primers with all three possible anchored bases (ACGTAATACGACTCACTATAGGGCGAATTGGG-TCGACTTTTTTTTTTTTTTTTTTn1 wherein n1=A/C or G) (SEQ ID NO: 3) along with other components for first-strand synthesis reaction except reverse transcriptase. This mixture was incubated at 65°C for five minutes, chilled on ice and the process repeated.

Alternatively, the reaction mixture may include 10 µg of total RNA, and two pmol of one of the two base anchored oligo(dT) primers a heel such as RP5.0 (CTCTCAAGGATCTTACCGCTT₁₈AT) (SEQ ID NO: 4), or RP6.0 (TAATACCGCGCCACATAGCAT₁₈CG) (SEQ ID NO: 5), or RP9.2 (CAGGGTAGACGACGCTACGCT₁₈GA) (SEQ ID NO: 6) along with other components for first-strand synthesis reaction except reverse transcriptase. This mixture was then layered with mineral oil and incubated at 65°C for seven minutes followed by 50°C for another seven minutes. At this stage, 2 µl of Superscript reverse transcriptase (200 units/µl; GIBCO/BRL) was added quickly and mixed, and the reaction continued for one hour at 45-50°C. Second-strand synthesis was performed at 16°C for two hours. At the end of the reaction, the cDNA was precipitated with ethanol and the yield of cDNA calculated. In our experiments, ~200 ng of cDNA was obtained from 10 µg of total RNA.

The adapter oligonucleotide sequences were as follows:

A1 (TAGCGTCCGGCGCAGCGACGGCCAG) (SEQ ID NO: 7) and A2 (GATCCTGGCCGTCGGCTGTCTGTTCGGCGC) (SEQ ID NO: 8). One μ g of oligonucleotide A2 was first phosphorylated at the 5' end using T4 polynucleotide kinase (PNK). After phosphorylation, PNK was heated denatured, and one μ g of the

5 oligonucleotide A1 was added along with 10 \times annealing buffer (1 M NaCl/100 mM Tris-HCl (pH 8), 10 mM EDTA (pH 8) in a final volume of 20 μ l. This mixture was then heated at 65°C for ten minutes followed by slow cooling to room temperature for thirty minutes, resulting in formation of the Y adapter at a final concentration of 100 ng/ μ l. About 20 ng of the cDNA was digested with four units of BglII in a final volume of 10 μ l for thirty minutes

10 at 37°C. Two μ l (4 ng of digested cDNA) of this reaction mixture was then used for ligation to 100 ng (50-fold) of the Y-shaped adapter in a final volume of 5 μ l for sixteen hours at 15°C. After ligation, the reaction mixture was diluted with water to a final volume of 80 μ l (adapter ligated cDNA concentration, 50 pg/ μ l) and heated at 65°C for ten minutes to denature T4 DNA ligase, and 2 μ l aliquots (with ~100 pg of cDNA) were used for PCR.

15 The following sets of primers were used for PCR amplification of the adapter ligated 3' -end cDNA: TGAAGCCGAGACGTCGGTCG(T)₁₈ n1, n2 (SEQ ID NO: 9) (wherein n1, n2 = AA, AC, AG, AT, CA, CC, CG, CT, GA, GC, GG and GT) as the 3' primer with A1 as the 5' primer or alternatively RP-5.0, RP 6.0, or RP 9.2 used as 3' primers with primer A1.1 serving as the 5' primer. To detect the PCR products on the display gel, 24 pmol of

20 oligonucleotide A1 or A1.1 was 5'-end-labeled using 15 μ l of [γ ³²P]ATP (Amersham; 3000 Ci/mmol) and PNK in a final volume of 20 μ l for thirty minutes at 37°C. After heat denaturing PNK at 65°C for twenty minutes, the labeled oligonucleotide was diluted to a final concentration of 2 μ M in 80 μ l with unlabeled oligonucleotide A1.1. The PCR mixture (20 μ l) consisted of 2 μ l (100 pg) of the template, 2 μ l of 10 \times PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 μ l of 15 mM MgCl₂ to yield 1.5 mM final Mg²⁺ concentration

25 optimum in the reaction mixture, 200 μ M dNTPs, 200 nM each 5' and 3' PCR primers, and 1 unit of Amplitaq Gold®. Primers and dNTPs were added after preheating the reaction mixture containing the rest of the components at 85°C. This "hot start" PCR was done to avoid non-specific amplification arising out of arbitrary annealing of PCR primers at lower temperature

during transition from room temperature to 94°C in the first PCR cycle. PCR consisted of five cycles of 94°C for thirty seconds, 55°C for two minutes, and 72°C for sixty seconds followed by twenty-five cycles of 94°C for thirty seconds, 60°C for two minutes, and 72°C for sixty seconds. A higher number of cycles resulted in smeary gel patterns. PCR products (2.5 µl) were analyzed on 6% polyacrylamide sequencing gel. For double or multiple digestion following adapter ligation, 13.2 µl of the ligated cDNA sample was digested with a secondary restriction enzyme(s) in a final volume of 20 µl. From this solution, 3 µl was used as template for PCR. This template volume of 3 µl carried ~ 100 pg of the cDNA and 10 mM MgCl₂ (from the 10× enzyme buffer), which diluted to the optimum of 1.5 mM in the final PCR volume of 20 µl. Since Mg²⁺ comes from the restriction enzyme buffer, it was not included in the reaction mixture when amplifying secondarily cut cDNA. Individual cDNA fragments corresponding to mRNA species were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Bands were extracted from the display gels as described by Liang *et al.*, (Cur. Opin. Immunol. (1995) 7, 274-280), re-amplified using the 5' and 3' primers, and subcloned into pCR-Script with high efficiency using the PCR-Script cloning kit from Stratagene (La Jolla, CA). Plasmids were sequenced by cycle sequencing on an ABI automated sequencer. Alternatively, bands were extracted (cored) from the display gels, PCR amplified and sequenced directly without subcloning.

cDNA CAP is a band that corresponds to a cDNA derived from an mRNA species that is expressed in myocardial biopsies obtained from patients prior to LVAD implantation and following cardiectomy prior to cardiac transplantation (Figure 1). The band corresponding to this cDNA was sequenced.

The nucleotide sequence of the full-length cDNA corresponding to the differentially regulated CAP band is set forth in SEQ ID NO: 1. The cDNA comprises 1990 base pairs with an open reading frame from nucleotides 283-1356 encoding a protein of 358 amino acids. The amino acid sequence is presented in SEQ ID NO: 2. The predicted isoelectric point of CAP is approximately 12.2.

Example 2: Quantitative RT-PCR Analysis of CAP Expression

Real time RT-PCR detection was accomplished by the use of the ABI PRISM 7700 Sequence Detection System. The 7700 measures the fluorescence intensity of the sample each cycle and is able to detect the presence of specific amplicons within the PCR reaction.

5 Each sample was assayed for the level of GAPDH and Clone 14431. GAPDH detection was performed using Perkin Elmer part#402869 according to the manufacturer's directions. Primers were designed for clone 14431 using Primer Express, a program developed by Perkin Elmer to efficiently find primers and probes for specific sequences. These primers were used in conjunction with SYBR green (Molecular Probes), a nonspecific double stranded DNA
10 dye, to measure the expression level of a clone 14431, which was normalized to the GAPDH level in each sample.

The normalized values for CAP expression in myocardium prior to LVAD therapy and following cardiectomy prior to heart transplant are reported in Figures 2-4. In Figure 2, samples 214, 206, 207, 208, 323, 329, 558, and 562 are normal samples, the remainder are
15 pairs from patients who underwent LVAD therapy prior to cardiac transplantation. Of that remainder, samples 1744, 1754 and 1736 (LVAD core) are myocardial biopsies taken prior to LVAD implantation. Samples 1743, 1753 and 1731 (cardiectomy) are myocardial biopsies taken following cardiectomy prior to cardiac transplantation. An increase in CAP mRNA expression was observed in cardiectomy samples when compared to their matched core
20 samples in all three patients.

An expanded panel of samples from sixteen patients was analyzed for increases in CAP mRNA expression in myocardium following cardiectomy prior to heart transplant when compared to myocardium prior to LVAD therapy. An increase in 144331 mRNA expression following LVAD therapy was observed in myocardium of eleven of sixteen patients (Figure
25 3). An extended panel of samples from patients suffering from ischemic cardiomyopathy, idiopathic cardiomyopathy and valvular disease was also analyzed (Figure 4). A significant increase in CAP expression was observed in myocardial tissue from patients suffering from ischemic cardiomyopathy (IsCM), idiopathic cardiomyopathy (IDCM) and valvular disease (Valvular) when compared to normal myocardial tissue (Normal) (see inset table). When

results from the four groups were combined, CAP expression was observed to be significantly elevated in myocardial tissue (All Heart Failure).

Example 3: Quantitative PCR Expression Analysis of CAP mRNA

5 The tissue distribution of mRNA encoding the differentially regulated gene encoding the protein of CAP was analyzed by quantitative PCR expression analysis of RNA isolated from various tissues. RNA was isolated from human kidney, adrenal gland, pancreas, salivary gland, liver, prostate, thyroid, cerebellum, fetal brain, placenta, spinal cord, stomach, small intestine, bone marrow, thymus, spleen, heart, lung, testes, uterus, mammary gland and
10 trachea using standard procedures. PCR expression analysis was also performed using primers derived from SEQ ID NO: 1 using AmpliTaq Gold PCR® amplification kits (Perkin Elmer). The presence of variable levels of CAP mRNA was detected in several tissues other than the heart (Figure 5). CAP mRNA expression was most abundant in the spleen, testis and heart. Lower, but detectable levels, were observed in the small intestine, thyroid, adrenal
15 glands, fetal brain and prostate. Northern blots were also prepared to screen for CAP mRNA expression in various tissues (see Figure 7).

 Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following
20 claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

We claim:

1. An isolated nucleic acid molecule selected from the group consisting of: (a) an isolated nucleic acid molecule that encodes the amino acid sequence of SEQ ID NO: 2; (b) an
5 isolated nucleic acid molecule that encodes a fragment of at least 6 amino acids of SEQ ID NO: 2; (c) an isolated nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO: 1 under conditions of sufficient stringency to produce a clear signal; and (d) an isolated nucleic acid molecule which hybridizes to a nucleic acid molecule that encodes the amino acid sequence of SEQ ID NO: 2 under conditions of sufficient stringency
10 to produce a clear signal and (e) an isolated nucleic acid molecule that exhibits at least about 60% nucleotide sequence identity over nucleotides 283-1356 of SEQ ID NO: 1.
2. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule comprises the sequence of SEQ ID NO: 1.
15
3. The isolated nucleic acid molecule of claim 2, wherein the nucleic acid molecule consists of the sequence of SEQ ID NO: 1.
4. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid molecule
20 comprises nucleotides 271-1347 of SEQ ID NO: 1.
5. The isolated nucleic acid molecule of claim 2, wherein the nucleic acid molecule consists of nucleotides 271-1347 of SEQ ID NO: 1.
- 25 6. The isolated nucleic acid molecule of any one of claims 1-5, wherein said nucleic acid molecule is operably linked to one or more expression control elements.
7. A vector comprising an isolated nucleic acid molecule of any one of claims 1-5.

8. A host cell transformed to contain the nucleic acid molecule of any one claims 1-5.
9. A host cell comprising a vector of claim 7.
- 5 10. A host cell of claim 9, wherein said host is selected from the group consisting of prokaryotic hosts and eukaryotic hosts.
11. A method for producing a polypeptide comprising the step of culturing a host cell transformed with the nucleic acid molecule of any one of claims 1-5 under conditions in
10 which the protein encoded by said nucleic acid molecule is expressed.
12. The method of claim 11, wherein said host cell is selected from the group consisting of prokaryotic hosts and eukaryotic hosts.
- 15 13. An isolated protein or polypeptide produced by the method of claim 11.
14. An isolated protein or polypeptide selected from the group consisting of an isolated polypeptide comprising the amino acid sequence of SEQ ID NO: 2, an isolated polypeptide comprising a fragment of at least 6 amino acids of SEQ ID NO: 2, an isolated
20 polypeptide comprising conservative amino acid substitutions of SEQ ID NO: 2, an isolated naturally occurring amino acid sequence variants of SEQ ID NO: 2 and an isolated polypeptide exhibiting at least about 50% amino acid sequence identity with SEQ ID NO: 2.
15. An isolated antibody that binds to a polypeptide of either claim 13 or 14.
- 25 16. The antibody of claim 14 wherein said antibody is a monoclonal or polyclonal antibody.
17. A method of identifying an agent which modulates the expression of a

polypeptide of either claim 13 or 14 comprising the steps of:

- (a) exposing cells which express the polypeptide to the agent; and
- (b) determining whether the agent modulates expression of said polypeptide, thereby identifying an agent which modulates the expression of a polypeptide of either claim 13 or 14.

18. A method of identifying an agent which modulates the activity of a polypeptide of either claim 13 or 14 comprising the steps of:

- (a) exposing cells which express the polypeptide to the agent; and
- (b) determining whether the agent modulates the activity of said polypeptide, thereby identifying an agent which modulates the activity of a polypeptide of either claim 13 or 14.

19. The method of claim 18, wherein the agent modulates one activity of the polypeptide.

20. A method of identifying an agent which modulates the transcription of the nucleic acid molecule of any one of claims 1-5 comprising the steps of:

- (a) exposing cells which transcribe the nucleic acid to the agent; and
- (b) determining whether the agent modulates transcription of said nucleic acid, thereby identifying an agent which modulates the transcription of the nucleic acid molecule of any one of claims 1-5.

21. A method of identifying binding partners for a polypeptide of either claim 13 or 14 comprising the steps of:

- (a) exposing said protein to a potential binding partner; and
- (b) determining if the potential binding partner binds to said polypeptide, thereby identifying binding partners for the polypeptide.

22. A method of modulating the expression of a nucleic acid encoding a polypeptide

of either claim 13 or 14 comprising administering an effective amount of an agent which modulates the expression of a nucleic acid encoding the protein.

5 23. A method of modulating at least one activity of a polypeptide of either claim 13 or 14 comprising the step of administering an effective amount of an agent which modulates at least one activity of the protein.

10 24. A non-human transgenic animal modified to contain a nucleic acid molecule of any of claims 1-5.

25. The transgenic animal of claim 24, wherein the nucleic acid molecule contains a mutation that prevents expression of a functional protein.

15 26. A method of diagnosing cardiomyopathy in a subject, comprising the step of determining the level of expression of the nucleic acid or polypeptide of any one of claims 1-5 or 13-14.

20 27. A method of preventing or treating cardiomyopathy comprising administering a therapeutically effective amount of the nucleic acid or polypeptide of any one of claims 1-5 or 13-14.

25 28. The method of claim 27 wherein the treatment diminishes the occurrence of at least one of the following symptoms associated with cardiomyopathy; reduced ejection fraction, increased left ventricular diastolic dimension, decreased ventricular wall thickness, increased atrial size, valvular regurgitation, exertional intolerance and ventricular tachyarrhythmia.

29. A method of assessing the effect of LVAD treatment of cardiomyopathy comprising measuring the amount of a nucleic acid molecule of any one of claims 1-5

expressed wherein an alteration in the amount of said nucleic acid molecule expressed correlates to successful LVAD treatment.

5 30. A method of prolonging the amount of time a patient can remain on LVAD treatment by modulating the amount of a polypeptide of any one of claims 13 or 14 expressed in myocardial tissue.

10 31. A method of reducing the amount of time a patient remains on LVAD treatment comprising modulating the amount of a polypeptide of any one of claims 13 or 14 expressed in myocardial tissue.

32. A method of identifying agent(s) which modulate the amount of gene product from SEQ ID NO: 1 expressed comprising the steps of:

15 (a) administering the agent to be tested to a mammal; and
(b) measuring the amount of gene product from SEQ ID NO: 1 expressed in the tissue of said mammal;

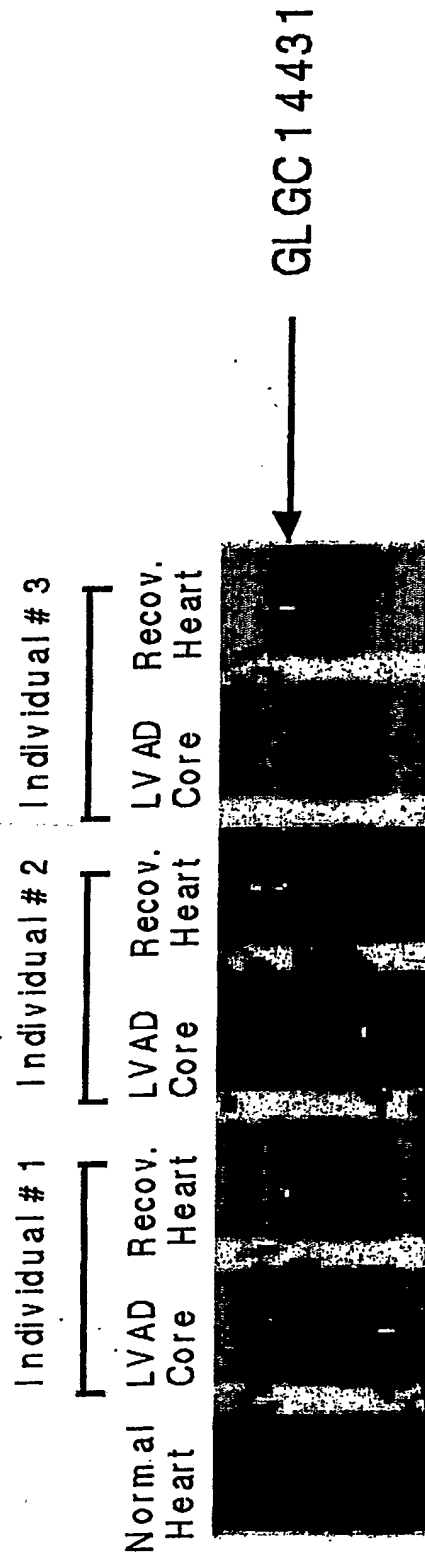
wherein an increase or decrease in the level of gene product from SEQ ID NO: 1 expressed indicates an agent capable of modulating expression of said gene product.

20 33. A method of preventing or treating cardiomyopathy comprising modulating the amount of gene product from SEQ ID NO: 1 expressed in myocardial tissue by administering a therapeutically effective amount of the agent identified in claim 32.

25 34. A method of identifying patients who require LVAD treatment by measuring the amount of gene product from SEQ ID NO: 1 expressed in myocardial tissue wherein an alteration in the amount of said gene product expressed is indicative of a patient requiring LVAD treatment.

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FIG. 1



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FIG. 2

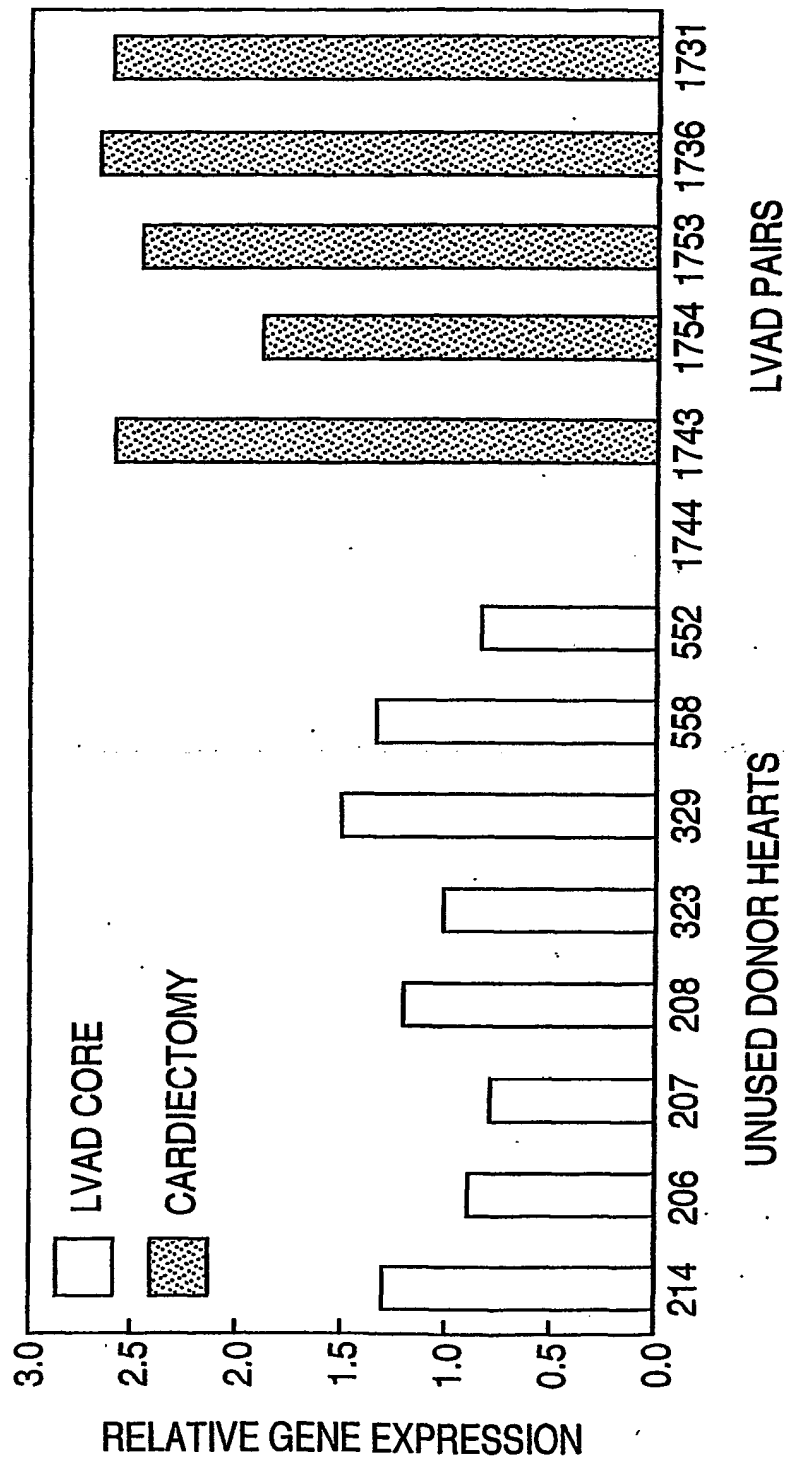


FIG. 3

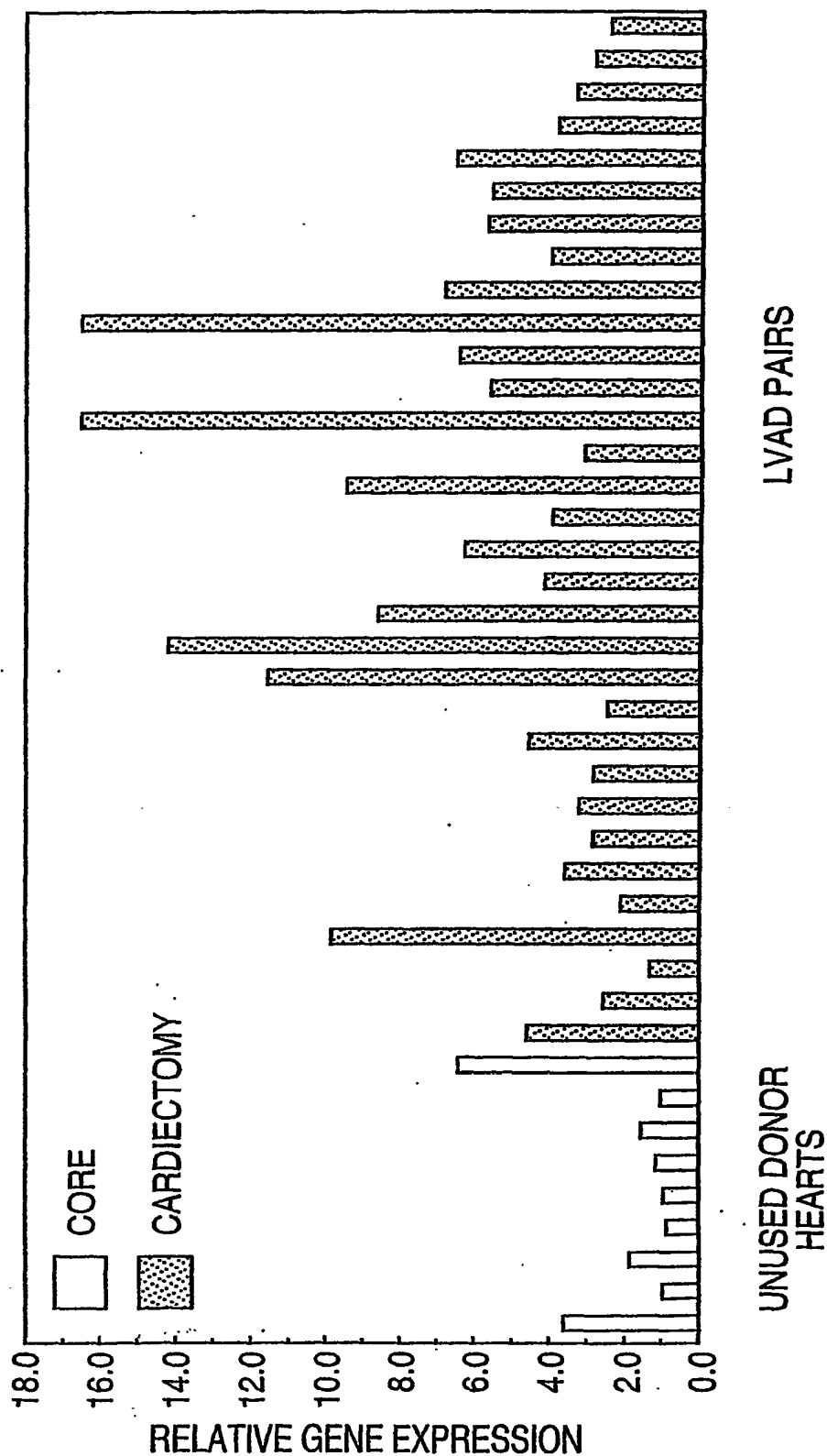
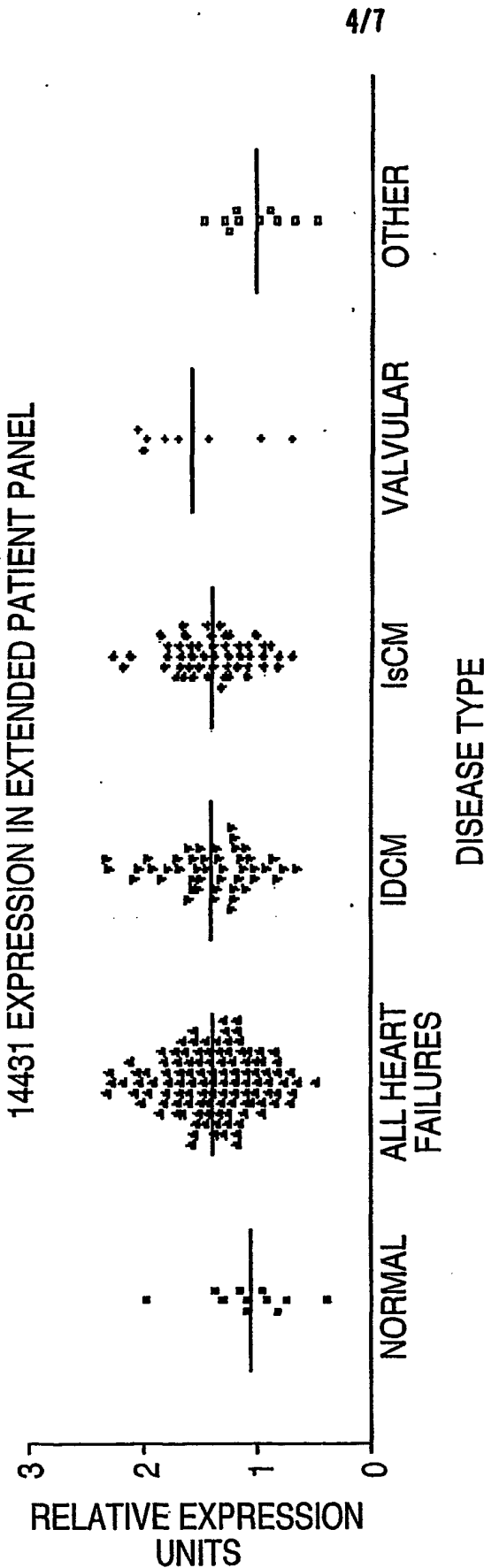


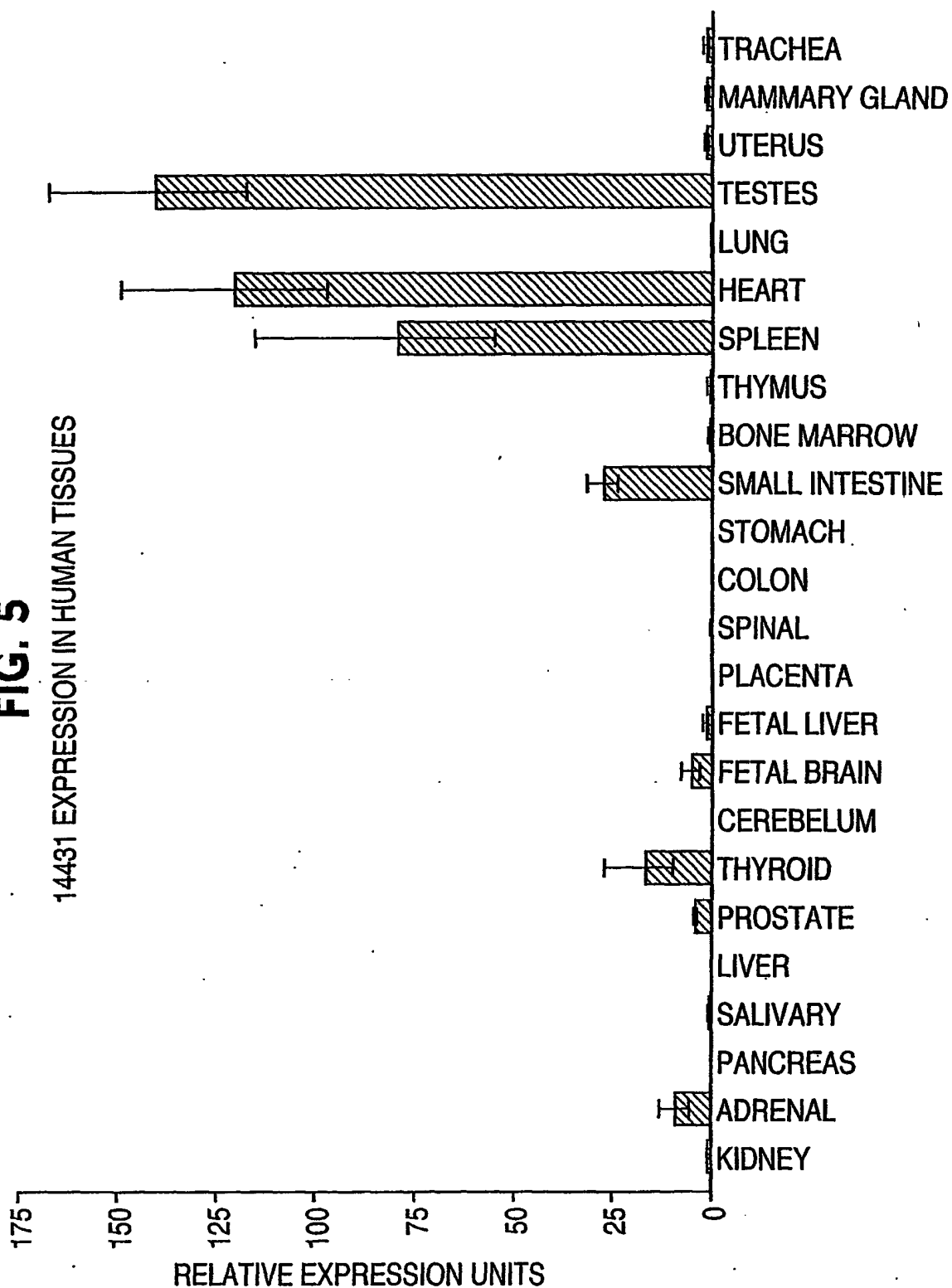
FIG. 4



PARAMETER	VALUE	DATA SET-B	DATA SET-C	DATA SET-D
DUNNETT'S MULTIPLE COMPARISON TEST	MEAN DIFF.			95% CI OF DIFF
NORMAL VS ALL HEART FAILURES	-0.3274	2.710	P < 0.05	-0.6340 TO -0.02079
NORMAL VS IDCM	-0.3436	2.675	P < 0.05	-0.6695 TO -0.01759
NORMAL VS ISCM	-0.3471	2.728	P < 0.05	-0.6699 TO -0.02420
NORMAL VS VALVULAR	-0.5261	2.959	P < 0.05	-0.9774 TO -0.07487
NORMAL VS OTHER	0.02416	0.1445	P > 0.05	-0.4002 TO -0.4485

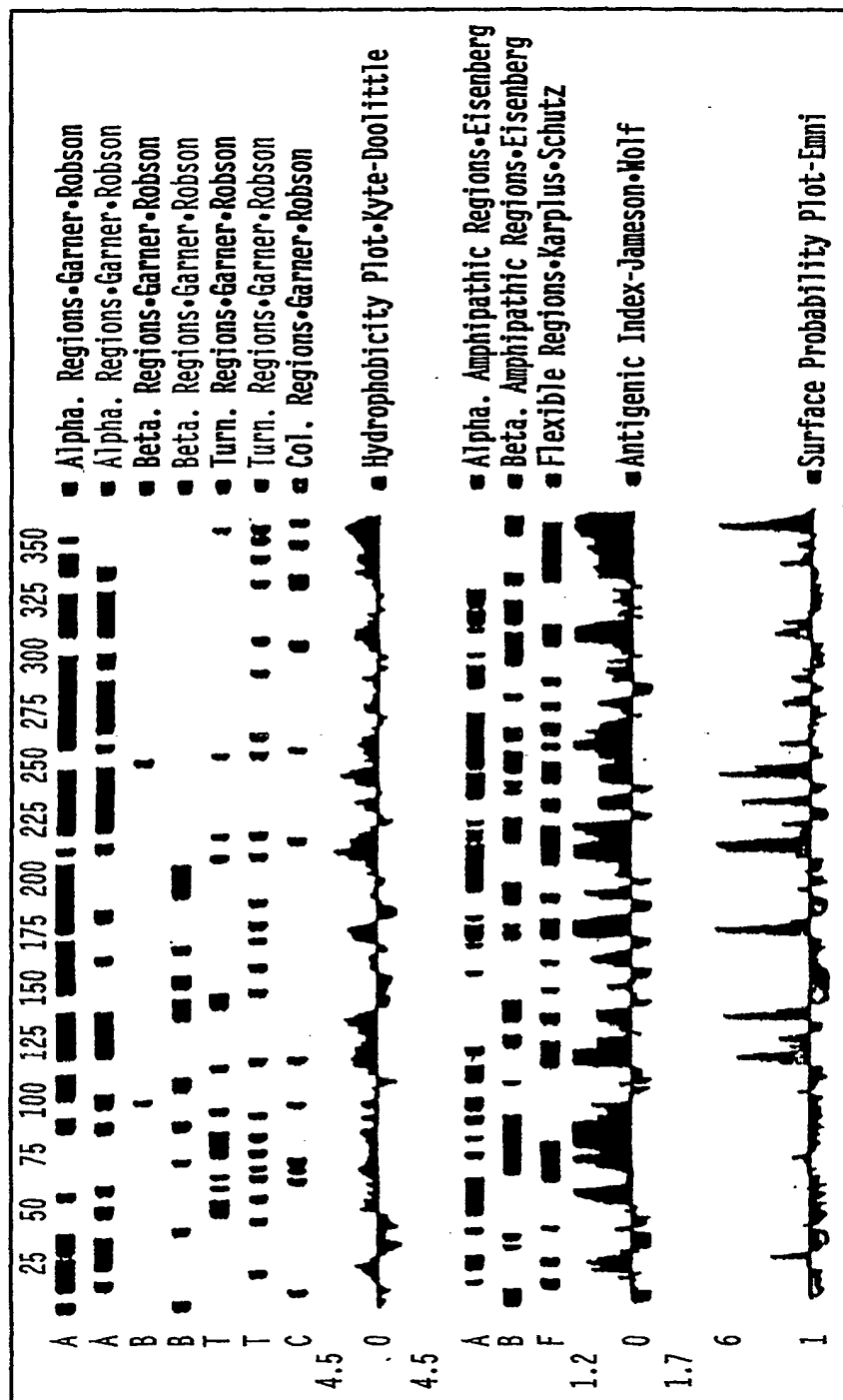
5/7

FIG. 5
14431 EXPRESSION IN HUMAN TISSUES



SUBSTITUTE SHEET (RULE 26)

FIG. 6



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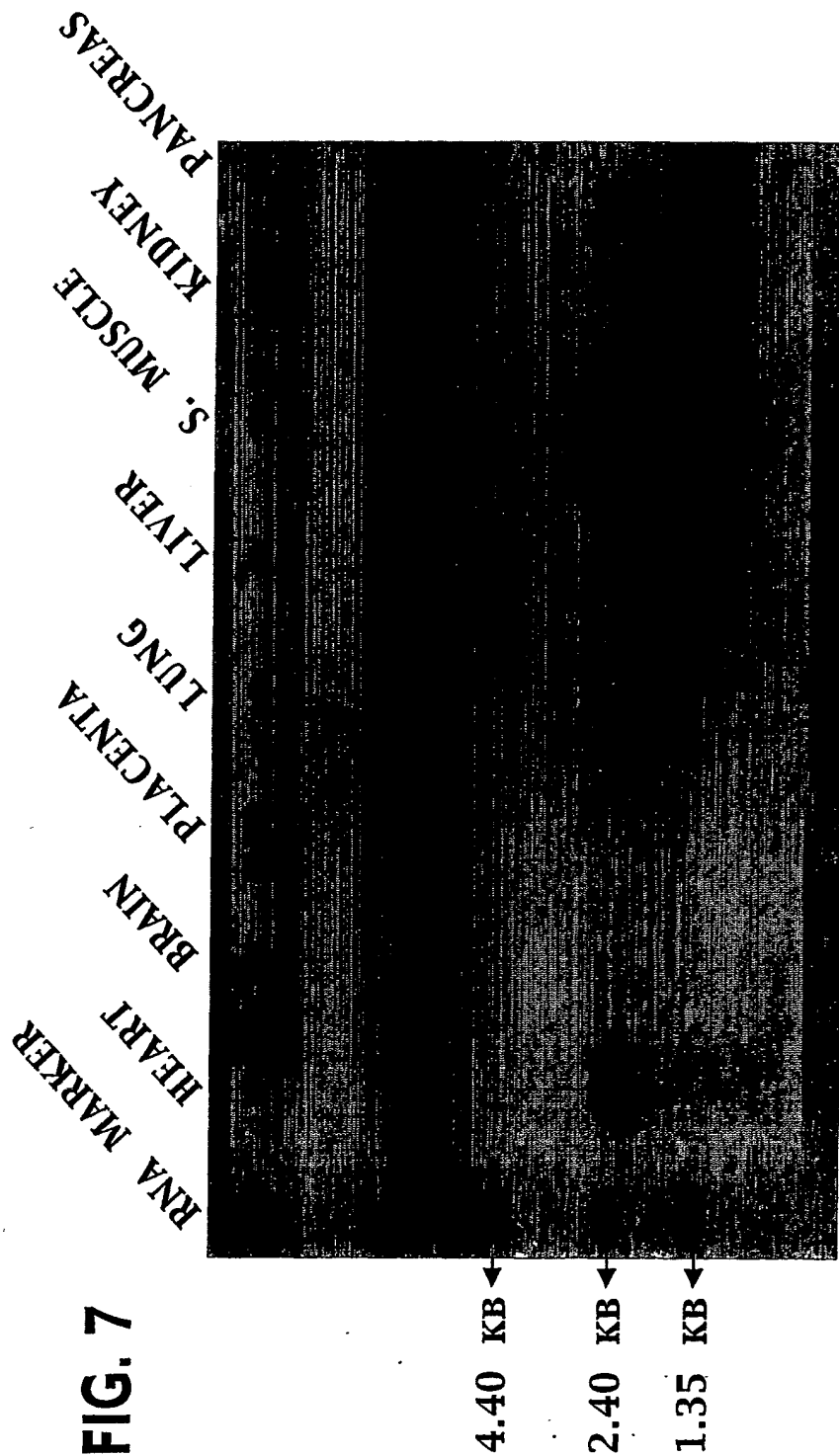


FIG. 7

SEQUENCE LISTING

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White, Margaret

<120> Identification of a cDNA Associated with Ventricular
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/05888

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 17/00; C07K 14/00; C12P 21/06

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/530, 387.1; 435/69.1, 320.1, 325, 252.3, 6, 7.1; 514/2; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	SPENCER et al. Regulation of microtubule dynamics and myogenic differentiation by MURF, a striated muscle RING-finger protein. J. Cell Biology. 21 August 2000, Vol. 150, No. 4, pages 771-784, see especially the Materials and Methods section and the sequence alignment attached.	1, 6-16, 20-22
A, P		2-5, 17-19, 23-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

09 MAY 2001

Date of mailing of the international search report

31 MAY 2001

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

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CHEMICAL MATRIX

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/05888

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536/23.1; 530/530, 387.1; 435/69.1, 320.1, 325, 252.3, 6, 7.1; 514/2; 800/2